EXHIBIT B

Nitrosamines as Impurities in Drugs—
Health Risk Assessment and Mitigation Public Workshop

March 29–30, 2021

Office of New Drugs,

Food and Drug Administration

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Expert Panelists

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PURPOSE AND GOALS OF THE WORKSHOP

The Office of New Drugs in the Center for Drug Evaluation and Research (CDER) of the Food and Drug Administration (FDA) organized this public workshop. International and national experts on nitrosamines were invited to discuss the chemistry and toxicology of nitrosamines in the environment and those recently identified as contaminants in pharmaceuticals. The experts answered and deliberated over 2 days on questions prepared by the FDA, and identified gaps in knowledge and the research needed to address and mitigate the drug contamination issue to ensure the safety of the drugs available to the American public.

For reasons of transparency, a recording and transcript of the workshop are available to the public on the FDA link. The background and a historical perspective on nitrosamines were presented by Dr. Gerhard Eisenbrand, a pioneer in nitrosamine research, and Dr. Sruthi King of the FDA described the incident of nitrosamine contamination in drugs.

INTRODUCTION AND QUESTIONS

Detection of nitrosamines as impurities in drugs was first reported in June 2018 by the Office of Generic Drugs—N-nitrosodimethylamine (NDMA) was found in valsartan, an angiotensin II receptor blocker (ARB). Since then, nitrosamines have been found in other ARBs, such as Irbesartan and Losartan; in other drug classes, such as antibiotics and antacids; and in a widely used diabetes drug, in which they were present in the active pharmaceutical ingredient (API) and/or the drug product (DP). As investigation into the contamination incident continued, multiple nitrosamines were identified within the various DPs.

In July 2018, Dr. Janet Woodcock, CDER Director, activated the CDER Nitrosamine Task Force (NTF) to manage the contamination incident, under the direction of the CDER Office of Counter Terrorism and Emergency Coordination (CTECS). Over 100 subject-matter experts from across CDER and other FDA Centers meet regularly to discuss and propose recommendations to mitigate the risk of nitrosamines in DPs and maintain patient access to critical medications. The NTF also discusses and proposes recommendations and updates senior management on strategies to mitigate risk and maintain patient access. The FDA has engaged international regulators to discuss and harmonize approaches for addressing nitrosamine contamination. To date, the following nitrosamines have been reported as impurities in drugs: NDMA, N-nitrosodiethylamine (NDEA), N-nitrosomethylphenylamine (NMPA), N-nitrosodiisopropylamine (NDIPA),

N-nitrosoisopropylethylamine (NIPEA), nitrosodibutylamine (NDBA), and N-nitroso-N-methyl-4-aminobutyric acid (NMBA).

Although almost 3 years have elapsed since the contamination incident, the FDA and other international regulatory agencies continue to face many challenges. One is that nitrosamines are formed and widely distributed in the environment—found in fresh vegetables, fruits, smoked and grilled meats and fish; in water; in the air; and formed endogenously. Therefore, eliminating or minimizing nitrosamines in drugs is problematic. Eliminating or minimizing nitrosamines as impurities in drugs begins with quality control. To date, not all root causes have been identified; new root causes continue to emerge. Factors such as stability, excipients, and storage conditions affect nitrosamine formation, hampering mitigation and control efforts.

Another issue is development and validation of highly sensitive analytical methods for detecting low levels of nitrosamines. In addition, the safety and cancer risk of nitrosamines in drugs must be determined taking into consideration drug shortages, disease condition, and patient population, as well as the resources needed to mitigate the contamination. Cancer risk assessment and determination of nitrosamine Acceptable Intake (AI), is complex. Nitrosamines are one of the few chemicals in the Cohort of Concern (CoC) in the International Council for Harmonisation (ICH) M7, a class of highly potent mutagenic carcinogens that requires strict controls to limit their amounts. Cancer risk assessment is based on lifetime exposure calculated using an extrapolated increase of 1 case of cancer in 100,000 people, which is considered an acceptable conservative level for risk. Because nitrosamines are potent carcinogens, they were studied extensively in the 1960s; >300 nitrosamines were tested in rodent cancer bioassays and >90% were found to be carcinogenic. The mechanism of action (MOA) of cancer induction is not fully understood. Structure-activity relationships have helped to identify structural properties responsible for carcinogenicity. Nitrosamines cause tumors in various organs and tissues; for example, liver, lung, nasal cavity, esophagus, pancreas, stomach, urinary bladder, colon, kidneys, and central nervous system.

Generally, nitrosamines are bioactivated to reactive intermediates, which interact with cellular targets and cause DNA damage; this process is essentially similar in animals and human. In contrast to nitrosamines requiring metabolic activation, direct acting N-nitroso compounds do not require bioactivation but directly bind to and alkylate DNA, thus causing mutations and eventually cancer. The unclear MOA, complex chemistries,

and the lack of robust carcinogenicity data or for some nitrosamines no data, complicate the determination of risk.

The FDA has been communicating risk information to the public since identification of the contamination incident. The FDA has informed industry and stakeholders on acceptable analytical methods; alerted patients, pharmacies, and care providers; recalled products; addressed media concerns, citizen petitions, and Congressional inquiries; and engaged in global communication and collaboration. The FDA published a Nitrosamine Guidance that provides information on quality and risk assessment. This FDA public workshop was a venue for public discussion of scientific issues by national and international expert panelists who identified critical gaps in knowledge that is needed to better inform of risk and to maintain safe drugs on the US market.

Seven questions were prepared by the FDA and posed to the expert panelists. They covered the topics of pharmaceutical quality manufacturing and control, environmental exposure to nitrosamines, their endogenous formation, pharmacokinetics, reactivity, biomarkers of exposure, Quantitative Structure-Activity Relationships ((Q)SAR), single and multiple nitrosamines in drug substance (DS) and/or DP, the Less-Than Lifetime (LTL) approach and duration of exposure, cancer risk and risk assessment, and identification of gaps in research to characterize cancer risk from exposure to nitrosamines present as impurities in drugs.

The questions and the comments of the expert panelists are provided below. They are followed by an overall summary and conclusion of the workshop.

Q1. What are the endogenous levels of nitrosamine formation in human and rodents? Once formed, what is the rate/kinetics of elimination? What are the conversion rates in the liver, circulation levels in blood, and normal variations? If this information is not available, can it be determined experimentally?

In addition to their abundance in the environment, nitrosamines are formed endogenously. To calculate risk, it is imperative to determine endogenous formation and understand the pharmacokinetics of nitrosamine formation and distribution. At this time there is a considerable gap in knowledge on the endogenous formation of nitrosamines in general, and NDMA in particular. It is unknown whether endogenous formation of carcinogenic nitrosamines exceeds, is equal to, or is less than, the levels detected in pharmaceuticals.

The expert panelists emphasized the importance of determining endogenous formation for assessing risk and recommended that experimental work be initiated as soon as possible. Some of the expert panelists stated that irrespective of the risk of endogenous exposure, exogenous nitrosamines pose an additional risk, and it is this incremental increase in risk that must be understood and investigated.

Much information is available in the literature on nitrosoproline, a nitrosamine that is not metabolized and is not mutagenic nor carcinogenic. Subjects dosed with proline plus nitrite or nitrate excreted enhanced levels of urinary nitrosoproline. The dose-response relationship for the formation of nitrosoproline in rats in vivo, after concurrent administration of various concentrations of the precursors, L-proline and sodium nitrite, revealed the logarithm of the amount of nitrosoproline formed to be proportional to the logarithm of the product of the proline dose and the square of the nitrite dose (Ohshima,Bartsch,1994, IARC SciPub 59,233-246).

However, reliable data on many reactive carcinogenic nitrosamines, e.g., NDMA and NDEA, are sparse because they are rapidly metabolized and the distribution and excretion of their metabolites are unclear. It is assumed however, that NDMA and NDEA are formed endogenously as their dietary precursors (dimethylamine and diethylamine, respectively), which, together with nitrites and nitrates, are present in foods. However, no quantitative assessment of NDMA or NDEA is available because of their rapid metabolism.

NDMA and many other nitrosamines are mainly metabolized by cytochrome P450 (CYP)2E1, which is subject to competitive inhibition by the simultaneous presence of other substrates for this enzyme. Therefore, it is important to consider the multiple factors that can alter tissue/organ metabolism, distribution, and excretion of nitrosamines and must be considered in risk assessments. Only rough estimates are available in the literature on endogenous formation of NDMA based on its detection in blood and urine; because of its rapid metabolism, only a small fraction is excreted in urine unchanged.

A potential approach is to measure alkylated DNA adducts formed in subjects. However, this is controversial because of the uncertainty over repair capacity (e.g., by the protein O^6-methylguanine-DNA-methyltransferase (MGMT), which repairs stoichiometrically; and by a suicide mechanism, O^6-methylguanine, the major precarcinogenic DNA adduct formed by NDMA), and the lack of knowledge on the sources of these adducts. Most

research in human has been small pilot studies that have several deficiencies. Three studies conducted over 10 years analyzed around 1000 blood samples from pregnant women with general environmental exposure. Promutagenic O^6-methylguanine adducts were detected in about 700 samples at a mean of 16 attomoles/µg DNA (range 4.5–10^9 attomoles/µg DNA; 1 attomole is 10^{-18} moles). Expressing adducts based on *cellular units* allows comparison with cellular MGMT content across tissues. Oral administration of NDMA at *low doses* to animals resulted in the highest adduct levels in blood. The MGMT content of primary human tissues is at least one order of magnitude higher than the highest adduct level in human blood DNA, therefore, loss of DNA repair as a result of MGMT depletion is unlikely to occur at background nitrosamine exposure levels (exogenous and endogenous).

How likely is NDMA to be the source of these adducts? Data from rodents exposed to a number of methylating compounds showed NDMA at low doses to be an efficient generator of O^6-methylguanine in blood leukocytes. Therefore, taking into account the methylating agents to which humans are exposed, it is reasonable to conclude that these O^6-methylguanine adducts in human tissue are mainly due to NDMA.

Studies in rodents and patas monkeys using low non-MGMT-depleting doses of NDMA showed comparable dose response curves for adduct accumulation in blood across species (slopes within a factor of 5), whereas the rates of repair of O^6-methylguanine in blood were similar in rat, monkey, and human (the latter from methylating drugs). The dose response of the steady-state level of O^6-methylguanine during chronic NDMA exposure of rat is linear, and the chronic exposures corresponding to the adduct levels found in human blood DNA corresponded to 982 µg/day (maximum) or 144 µg/day (mean background level of O^6-methylguanine) (see slides by Dr. Kyrtopoulos). This exposure is higher than estimated from exogenous exposure; therefore, it can be assumed to be of endogenous origin.

These estimates are comparable with those of human background NDMA exposures derived by alternative methodologies based on the concentration of NDMA circulating in human blood or excreted in urine. The adduct analytical methods employed in the above-mentioned human studies have potential flaws and so those studies need to be repeated using appropriate analytical methods. Additionally, there is uncertainty regarding the similarity of the dose-response curves between animals and human and it is important to understand the various factors that play a role in endogenous formation of NDMA. To date, no experiments have been conducted to investigate these factors.

Amounts based on exogenous exposure from real-life 'normal' consumption of food and medicines or the environment should also be determined to provide perspective on exposure from endogenous formation. The expert panelists agreed that exposure from endogenous formation of nitrosamines is likely to be higher than exposure from exogenous sources such as food. In general, exposure from food is estimated to be <1 µg per day across countries and cultures. Acknowledging the critical need to the determination of endogenous formation is a long-term goal for the current urgent status to assess risk of drugs contaminated with nitrosamines, other approaches should be considered. Examples include physiologically based pharmacokinetic (PBPK) modelling, measurement of O^6- methylguanine adducts as biomarkers of exposure (not risk; see the relevant discussion below), and read-across combined with (Q)SAR.

Q2. Can nitrosamines be classified? If yes, what is the basis of their classification? For example, could they be classified based on:

- **Carcinogenic potency?**
- **Chemical structure, e.g., aliphatic vs. cyclic?**
- **Chemical reactivity? Direct alkylating agents vs. indirect (require metabolism)? Adduct formed, e.g., O^6-, N^7-methylation?**
- **Other?**
- **Why would you choose this basis of classification?**

If classification is not possible, is it feasible to calculate a single acceptable intake (AI) value for nitrosamines; i.e., a class-specific limit, using the existing carcinogenicity study results of ≥110 nitrosamines (irrespective of study quality)?

More than 90% of known nitrosamines are carcinogens spanning several orders of magnitude of potency. They cause cancer in 40 animal species in multiple organs by different routes of administration (in some cases the same tumors via different routes). Also, the same nitrosamine can cause different tumors in different animal species and had different latencies following short (including a single dose) and long durations of exposure. This diversity hampers classification of nitrosamines. A panelist posed the following question: "should nitrosamines be classified?"

The panelists stated that nitrosamines can be classified, but several research gaps need first be addressed. A simple classification would be *carcinogens and non-carcinogens* based on existing data. However, classification based on carcinogenic potency, which is more informative, is complex and information is lacking. Using the 50% toxic dose (TD_{50})

values from the former Gold Carcinogenicity Potency Database (CPDB), this approach was collectively considered by the expert panelists to be the most appropriate in the absence of alternatives and can be revisited when some of the critical parameters have been addressed.

Alternatively, classification could be based on *chemical structure*. Some nitrosamines are devoid of carcinogenicity, such as those with tertiary butyl groups, nitrosated amino acids, or branched chains. Also, classification could be based on the likelihood/degree of *protonation* (PKb value), which reduces bioavailability and therefore bioactivation. Also, nitrosamines could be classified based on their *chemical reactivity*, an important factor, into *direct* vs. *indirect alkylating nitroso compounds.* For instance, nitrosoureas are direct alkylating agents and do not require metabolic activation.

The *Benchmark Dose–Lower (BMDL)*, which is based on the lower end of the dose response, e.g., 1 or 5%, and margin of safety were discussed as other bases for classification. The BMDL uses dose-response information whereas TD_{50} uses a one-dose estimate to extrapolate to a given risk level. Use of data from multiple doses increases the accuracy of extrapolation to lower doses. However, this is not available for all nitrosamines and BMDL calculations on the original data of nitrosamines have yet to be performed.

The consensus of the expert panelists was that generally BMDL is the preferred approach. However, for nitrosamines, the TD_{50} is the best-available carcinogenic risk estimation. (Q)SAR modelling based on TD_{50} values in conjunction with expert knowledge supplemented with empirical data as needed shows promise for classification (more on this in Q6). Several parameters must be considered in expert knowledge assessment, e.g., stereoelectronic considerations and their effects on alpha hydroxylation rates, the half-life of the intermediate diazonium ion, and the reactivity of the carbonium ion. Several of these parameters are based on predictions, reducing confidence in the model outcome. In addition, many of these models are computationally intensive and require highly trained staff.

A single value for an AI applicable to all nitrosamines was also discussed. For example, using the AI of the most potent nitrosamine or the median potency based on modeling and a 1:100,000 risk of cancer. The European Medicines Agency (EMA) has been using 18 ng/day based on a re-assessment of TD_{50} values by Lhasa/Leadscope. The difference

between 18 and 26.5 ng/day NDEA is small and such extrapolation may be acceptable until experimental data are collected to confirm or indicate the need for re-evaluation.

Nitrosamines could be classified based on the *range of TD_{50}s* (e.g., <1, 1–10, and 10–100 mg/kg); values >100 mg/kg could be discarded as being outside the hazard for drug contamination. The TD_{50} calculations have shortcomings because the studies had inconsistent designs, and exposure durations (some used a single dose) and involved a small number of animals and fewer than the optimal number of doses.

Classifying nitrosamines at this time is difficult and improbable because of lack of essential data such as on N-nitrosation reactivity, formation of DNA adducts, binding to DNA, and ADME in human and how it compares to that in animals. This can be tested by comparing O^6-alkylation or other mutagenic DNA damage induced by a nitrosamine in cancer target and non-target organs of rodents to that in corresponding human tissues/cells. Such an approach could use surrogate biomarkers, reporting relevant biotransformation steps such as alkylation of hemoglobin as a long-term biomarker (averaging 3–4 months of exposure) or of glutathione with subsequent excretion of mercapturic acids in urine as short-term biomarkers (exposure within the prior 48 h). This may even be possible at exposure levels associated with human exposure to drugs. If O^6-alkylation in human is less than in rodents this would indicate differential biotransformation that can be ascertained using advanced PBPK modelling.

Testing for mutagenicity by Ames test is the first step in determining the potential carcinogenicity of a compound. However, the mutagenic potencies of nitrosamines differ by four or five orders of magnitude and do not correlate to similar carcinogenic potency. For example, the bacterial mutagenicities of NDMA and NDEA are similar, however, the latter is a somewhat more potent carcinogen.

Reliable risk assessment requires knowledge on the mechanism of carcinogenesis, which is currently not fully understood; not all nitrosamines give rise to O^6-guanine methylation. If a chemical forms adducts with oxygens of DNA bases, such as O^6-methylguanine, it is likely to be a potent carcinogen. DNA adduct formation or induction of DNA damage may be used as markers of potency (or risk assessment), provided it can be reliably assessed in an ex vivo system which would allow comparative dosimetry of DNA damage. The same conclusion can be reached for direct-alkylating nitrosamines, such as nitrosoureas. However, for some nitrosamines detected as contaminants in drugs, the DNA adducts formed and their reactivity and stability are unknown.

Therefore, such nitrosamines cannot be classified. Until experimental data are available, these compounds are assessed based on the TD_{50} value of a surrogate molecule together with expert judgment on their metabolism and reactivity with DNA.

Another pivotal aspect of nitrosamines is their *reactivity*. There are few, if any, data on the correlation between reactivity and carcinogenic potency. However, reactivity can be measured using a sensitive and quantitative method, e.g., high-resolution mass spectrometry. Another classification approach is *Bayesian probabilistic modelling*, in which current data are combined with SARs and biomarker or metabolism data to assign a probability to prediction; such an approach could enable classification.

Another possibility is similar to the *threshold of toxicological concern (TTC)* paradigm used in ICH M7 but specific to nitrosamines, which are in the CoC based on the TTC and excluded from the Cramer classes.

Incorporation of safety factors in sensitive patient populations, e.g., children, pregnant women, and individuals deficient in metabolic enzymes, was also discussed. There are data showing transplacental carcinogenesis by nitroso compounds, which is a concern in pregnant women exposed to drugs contaminated with nitrosamines. Also unknown is the risk for individuals lacking or deficient in CYP450 and in other enzymes involved in biotransformation. The expert panelists suggested that default safety factors should be considered when determining risk.

In summary, reliable classification of nitrosamines is not possible without knowledge of their metabolism in human, efficiency of metabolic enzymes (rate, content in tissues and blood), human DNA repair capacity, MOA of cancer induction, level of reactivity, and the stability and DNA binding. At this time data are lacking or sparse for all of these parameters. In this situation, nitrosamines could be classified by relatively imprecise structure-based modelling, which is dependent on predictions.

Q3. The carcinogenic potential of nitrosamines is dose and duration dependent:

- **Is there an in vivo exposure level for nitrosamines to define low vs. high risk for carcinogenicity? Is it appropriate to calculate a no observed effect level (NOEL) dose for carcinogenicity? What are the criteria to do so (Ames negative, in vivo mutation assay negative, other)?**
- **Can LTL approach as described in ICH M7 be used to determine the AI of a nitrosamine if a drug is indicated for a short duration of use?**

Given that humans are exposed endogenously and exogenously to nitrosamines, the differences in DNA repair capacity among humans as well as among animal species, and the less than ideal quality of nitrosamine carcinogenicity studies, the expert panelists were asked if an in vivo exposure level that can define low vs. high risk for cancer could be identified and whether a NOEL dose could be established. Several published studies have shown a clear and abrupt transition of the dose-response to no toxicity, whereas other nitrosamines showed a gradual change with a curvilinear dose response and a sigmoidal curve at low doses. It is important to determine the dose rate, i.e., the interval between doses, and how it affects DNA repair.

Earlier studies showed that the cancer rate is independent on age. Indeed, when NDEA at the same doses was administered to animal species of different life expectancies, all animals developed tumors at the same rate and time. Issues over the applicability of the LTL approach (ICH M7) to nitrosamines include the reliability of the models for extrapolating from long to short durations, model sensitivity, and the shape of the dose-response curve (nonlinear vs. threshold). These are important points to consider when discussing the question of LTL. The expert panelists did not consider deriving a NOEL for nitrosamines to be appropriate, and it is not accepted for genotoxic carcinogens.

Reference was made to the Peto rat mega-bioassay (>4000 rats), in which the dose-response curve at low doses was linear with no threshold. This linearity was supported by the levels of DNA adducts. Therefore, cancer bioassay and adduct formation results produced a linear dose response without evidence of a threshold and so a NOEL was not identified. These findings were obtained in rodents; whether the paradigm holds in human is unclear.

A negative Ames should be followed-up by in vivo mutation tests, and a single in vitro mutation test is not adequate or acceptable to evaluate a nitrosamine. In vivo mutation tests do not predict in vivo cancer potency but are more physiologically relevant. Also, the duration of exposure and dose are important, but their determination is time- and resource-intensive. The linearity of the DNA adduct dose response may or may not induce mutation. Mutation induction requires several steps, some of which are toxic (e.g., cell death) whereas others proceed to mutation. There are also several steps between mutation and cancer, failure at any one of which halts carcinogenesis. Therefore, DNA adduct formation alone is inadequate to confirm mutation or cancer. Most nitrosamines are positive by standard Ames test, however, positivity is protocol dependent in some cases. NDMA was negative in rat S9 but positive in hamster S9. Also,

the chemical potency responses differ among rat, mouse, and hamster S9. Moreover, there are no such data using human liver S9.

It was reiterated that a negative Ames test for a nitrosamine does not suffice to qualify that nitrosamine, and very few nitrosamines are non-mutagenic and non-carcinogenic. Therefore, nitrosamines are typically considered guilty until proven innocent. An attendee asked why not test the nitrosamine impurity directly by in vivo mutation assay, skipping the Ames test? The expert panelists indicated that this would not be appropriate because most nitrosamines are positive, and the Ames test is rapid, reliable, in vitro, and inexpensive. In vivo tests involve animals, prolonged dosing, and are resource-intensive. Moreover, if only an in vivo test is performed and the result is negative, this alone cannot qualify a nitrosamine impurity. Negative Ames results can be due to the test not detecting all types of DNA damage, e.g., DNA deletions. Also, the in vitro metabolic system in the Ames test may not be efficient or adequate, and metabolic capacity is assessed using liver homogenates supplemented with nicotinamide adenine dinucleotide phosphate (NADPH); by contrast, in vivo exposure is to the intact liver.

If a nitrosamine is negative by standard or optimized (hamster S9, 30% S9) Ames test, an in vivo gene mutation test and an expert knowledge-based assessment must be conducted to determine intermediate nitrosation, reactivity, and stability. This, together with supporting evidence, such as established or expected (from SAR) absorption, distribution, metabolism, and excretion (ADME) behavior (e.g. nitrosated amino acids, which are excreted in urine), supports the premise that a non-mutagenic nitrosamine is unlikely to be carcinogenic.

Another query was what level of nitrosamine exposure is of de minimis cancer risk? This depends on the nitrosamine; there is *no generalization*. Certainly, milligram amounts are not accepted for NDMA or NDEA but could be accepted for nitrosoproline, which is not metabolized, mutagenic, or carcinogenic. Therefore, assessment would have to be case by case.

There was general consensus that the LTL approach could be applied to nitrosamines as impurities in drugs indicated for a short duration of use. Carcinogenesis is a function of accumulated dose and DNA damage; however, other factors (e.g., cell proliferation, apoptosis, metabolism, and repair) are also implicated. For DNA adduct formation, the dose response is linear over a wide range, therefore, high exposure for a short duration would have comparable risk to low exposure over a long duration because the

integrated overall lifetime exposure as it relates to DNA damage is the same. In addition, the 1:100,000 cancer risk is conservative and a small increase in risk over a short exposure period would be acceptable.

However, the dose response of the other factors implicated in carcinogenesis (proliferation, metabolic cofactors, DNA adducts), is unknown for high exposure over a short duration. Therefore, for nitrosamines the answer to the applicability of LTL is not as simple as stating that the effect of high exposure over a short duration is similar to low exposure over a long duration. DNA repair capacity must be considered for exposure to high doses of a nitrosamine for a short duration.

Moreover, total exposure to nitrosamines, i.e., the contents in food, which are low (≤1 µg/day), and all involved enzymes (e.g., MGMT, alpha hydroxylase) should be considered. The amounts of nitrosamines as impurities in drugs are considered low in the sense that depletion of MGMT is not expected. The consensus was that depletion of repair enzymes is unlikely at currently reported exposures, noting also that there are no known examples of MGMT-deficient human primary cells (the only such examples being cancer cells). There is, however, the caveat of individuals with repair deficiencies.

As indicated above, the experts cautioned using drugs contaminated with nitrosamines in children and recommended incorporating safety factors (SFs). ICH M7 does not include SFs and the FDA Center for Foods and Applied Nutrition (CFSAN) does not adjust for longer lifespan. The latter uses models to estimate consumption of a particular food item from surveys of dietary habits. The U.S. Environmental Protection Agency (EPA) does not assess carcinogenic impurities in fruits but uses SFs to determine the limits of pesticides used on foods. In short-duration cancer studies, neonatal mice had different responses than adults when dosed over a short duration. This is a cautionary note for using LTL for nitrosamines as impurities in drugs for children.

Reference was made to a study conducted in the 1960s (Druckrey et al.), which showed a linear no-threshold dose response over a dose range of 10 mg/kg bw to 70 µg/kg bw. The slopes were parallel down to the low dose and the lower end of the low dosage of 70 µg/kg bw; these tumor findings continued for the lifetime of the rats (3 years).

Q4. How would the risk assessment change when multiple nitrosamines are present in a drug product? What are the key variables to consider when conducting such risk assessments? (nonmutagenic carcinogen + mutagenic carcinogen; nonmutagenic carcinogen + weakly mutagenic carcinogen; multiple mutagenic carcinogens).

Multiple nitrosamines have been identified as impurities in a single DS and/or DP over the two-and-a-half years since the contamination incident was reported. It is unclear whether the PK and DNA adduct repair are affected, taking into consideration exposure from other nitrosamine sources and whether their effects are additive or synergistic. Adding multiple nitrosamines assumes similar mechanisms of metabolic activation and action, potencies, and reactivities, none of which is known. However, if there are adequate data (for both structural similarities and differences) to enable prediction by (Q)SAR then these parameters could be compared and data for similar nitrosamines could be grouped.

Nitrosamines with the same MOA may have different potencies; in such cases, the significance of adding a low-potency nitrosamine to a high-potency nitrosamine is unclear, i.e., the value of the high-potency chemical will be used. The expert panelists discussed the application of cumulative risk assessment to nitrosamines. The European Food and Safety Authority (EFSA) has conducted much work on residues from pesticides. For example, in mixtures whether there is a mutagenic or nonmutagenic chemical is irrelevant. If one of the chemicals is carcinogenic, that information would be the one used to calculate an AI, i.e., risk would be additive or data of the most-potent nitrosamine would be used to calculate AI. Mixtures of large bulky nitrosamines can result in competition for enzyme activation and repair. This is not an issue for low-molecular-weight nitrosamines, and so assumption of an additive effect may not be appropriate in such cases.

The need for up-to-date information on exogenous and endogenous formation of nitrosamines was reiterated by the expert panelists. Such information is essential for risk assessment and accurate calculation of AI. However, most available data are 20–50 years old and much has changed since, including dietary habits, exposures, and analytical methods. The levels of nitrosamines as impurities in drugs are likely miniscule in comparison to exogenous exposure from foods and even more so to endogenous levels. However, this estimate is based on old data and is thus likely to be inaccurate. By contrast, data on nitrosamines in food are more up-to-date and the food industry has made much improvement over the past few decades.

Recent studies on the nitrate, nitrite, and nitrosamine contents of foods have reported very small amounts; 0.2–0.4 µg/day and globally <1 µg/day for nitrosamines.

The expert panelists emphasized quality control via good manufacturing practices (GMP) and understanding the chemistry of each chemical of DS and DP for eliminating or minimizing nitrosamines in drugs (for more details, see Q7). Also, the drug formulation plays a role, e.g., polymorphic forms; amorphous vs. crystalline drug forms could contribute to the degree of degradation, making the product more stable. However, PK parameters may be affected when the formulation is changed to improve stability, e.g., ascorbic acid added to prevent formation of NDMA in aminopyrine. However, the former was protective only after the first passage and was excreted thereafter, and aminopyrine undergoes recirculation. Therefore, protection was lost after the first passage.

Several biomarkers of exposure and perhaps for risk assessment were discussed, including DNA adducts, MGMT, and metabolism modelling. The expert panelists were asked which is most suited for nitrosamine risk assessment. DNA adducts were deemed optimum because current analytical technologies (e.g., high-resolution mass spectroscopy) are highly sensitive and reliable. Many previously problematic artifacts can now be addressed. Prior studies involving nitrosoproline measured urinary levels following administration of proline and nitrite to human volunteers. Today, individuals could be exposed to labelled precursors of NDMA, i.e., dimethylamine labelled with C^{13} as a tracer, and the levels of C^{13}-labelled DNA adducts assayed.

However, using only DNA adducts as biomarkers for nitrosamine exposure is inappropriate because it requires knowledge of the MOA. DNA damage is critical for nitrosamine carcinogenicity and O^6-methylation is the relevant adduct for NDMA. DNA methylation as a mechanism of carcinogenesis has been extensively studied. However, not all nitrosamines methylate; therefore, O^6-methylation is not the sole MOA—other sites (e.g., O^4, N^7) and other mechanisms should also be studied. Our knowledge is inadequate to develop biomarkers for risk but these could be biomarkers of exposure because they can potentially be quantified by more-sensitive methods. Further work is needed to understand the MOA of nitrosamines before DNA adducts can be used as biomarkers for risk assessment.

Concerns were raised over quantification of DNA adducts for nitrosamines because of exposure and formation from food and endogenously; therefore, separation of adduct

sources is important. Completed and ongoing human studies have measured total nitrosamines in urine after administration of drugs contaminated with them or other sources. The results showed very high levels of NDMA but further investigations indicated that the analytical methods were flawed. This is an important point—for assessing exposure, one method does not fit all. The websites of the FDA and other regulatory authorities list appropriate analytical methods and conditions.

Q5. Should the regulatory limits for nitrosamines listed for food and water or, amount formed endogenously, be considered in determining AI of nitrosamines in drugs?

Patients take medicines to treat a disease and so anticipate benefit; in most cases it is not a choice. The American public expects their medicines to be safe. However, one chooses what to eat and drink. Based on earlier discussion, endogenous production of nitrosamines and their precursors is likely to constitute a more important source of exposure than exogenous intake.

To estimate total dietary intake, in addition to nitrosamines, calculation of nitrate and nitrite intake is critical. Indeed, this is so not only for diets rich in these compounds but across all types of diet, i.e., an unbiased assessment. The panelists agreed that intake from all sources should be considered in nitrosamine risk assessment and determination of AI. However, they emphasized again the need for accurate assessment of endogenous formation. If the data show endogenous formation higher by several orders of magnitude, calculating an AI would not be difficult from a numerical risk assessment perspective. Using the existing data on exogenous content and intake was referred to as a common-sense approach. Unlike endogenous exposure, there is much old and new information on exogenous exposure. These constitute a good database, but it needs to be validated using current data. The likelihood is that intake is similar at 0.2–0.5 µg/day and <1 µg/day for nitrosamines.

Also, determination of endogenous formation is very important. However, many factors, such as dietary habits and overall health (inflammation, infection), play a role. The argument for a no answer was that if exposure from all sources other than drug contamination is large, then the health risk from the small amount present in drugs becomes insignificant. However, if these other sources are considered background, particularly endogenous formation whether large or small, they should be acknowledged as contributing to the background cancer risk in the general population. As an example, based on the discussion and data shown by Dr. Kyrtopoulos, mean

endogenous formation of 144 µg/day would translate (based on Peto bioassay) to a ~ 1% cancer risk for NDMA as the background incidence (the overall lifetime cancer incidence in humans is 30–40%). Nevertheless, traditional and M7 cancer risk is based on 1:100,000, which is an incremental risk over that of cancer in the general population, i.e., the background incidence is ignored. In principle this is the more accurate and correct approach, i.e., to consider nitrosamine exposure as a result of its presence only in drugs. This is also based on the uncertainty of endogenous formation. If endogenous exposure is overestimated (i.e., it is not >100 µg/day), then incremental exposure from drug contamination becomes more significant "conceptually." Therefore, exposure from other sources should not be considered in risk assessment or AI calculations for nitrosamines in drugs. It was acknowledged that investigation of endogenous formation is a relatively long-term goal and a practical approach is urgently needed, therefore, flexibility should be exercised.

It was pointed out that the body sees a molecule as-is and does not distinguish its origin, i.e., foods or drugs. Therefore, it is cumulative exposure that contributes to the additivity of the response. There are good data for exogenous exposure but endogenous data are sparse and highly variable; therefore, studies need to be repeated using current sensitive methods. It is important not to provide a wide range of limits for food vs. drugs so as not to compromise public confidence in risk evaluation.

There was support for nitrosamine incremental risk assessment based only on their presence in drugs. This is because endogenously generated nitrosamines are likely carcinogenic. It is possible that a spontaneous tumor load of 30–40% could be due to endogenously formed nitrosamines. If so, then there is no baseline for accurate determination of the additional cancer risk from nitrosamine-contaminated drugs. However, the TD_{50}-based risk calculation is the best approach for evaluating endogenous and exogenous exposure. Multiple compounds other than nitrosamines also contribute to the spontaneous tumor load; e.g., endogenously formed formaldehyde and ethylene oxide.

Another consideration in risk assessment is poly-pharmacy; if more than one drug is contaminated with nitrosamines then the cumulative risk must be considered, as should the cost of and resources required for implementation. There are good arguments on each side, i.e., determine cancer risk based only on the amount in drugs or on total exposure to nitrosamines. The amounts of nitrosamines in drugs may not add much to the overall risk from all sources (exogenous + endogenous + drugs). However, in the

absence of supporting data, communication on risk should take these factors into consideration.

The expert panelists stated that the structures of nitrosamines should be included in the assessment of risk. Most available data are on NDMA and NDEA, but other nitrosamines have been identified in drugs as contaminants. Therefore, a blanket assessment considering all nitrosamines at the same level of risk is inappropriate because their biological effects could be different. Exposure to such nitrosamines should be avoided by implementing correct chemistries. It was emphasized that it should not be considered acceptable to be exposed to such low levels in drugs because we are exposed to them in food. Again, there are reliable data on nitrosamines in food that could be used as a reference for risk assessment.

Future availability of data on the magnitude of endogenous formation will enable accurate assessment of total exposure and risk. Nevertheless, GMP, correct chemistry (avoiding drug precursors that can be nitrosated), and mitigation can prevent nitrosamine contamination of drugs. Over 40 years ago, the World Health Organization (WHO) introduced the Nitrosation Assay Procedure (NAP) to evaluate the nitrosability of drugs, which could be used to prevent nitrosamine formation. GMP and mitigation are key, irrespective of whether a risk assessment is performed.

A comment was made on whether the older data can be used to determine endogenous formation of some nitrosamines and extrapolated to current information on dietary intake. For example, the extensive work on endogenous formation of nitrosoproline, which is not metabolized and is excreted unchanged. When nitrates (400 mg) and proline (500 mg), were given to people, the urinary yield for nitrosoproline was in nanomoles. This finding is similar to several 20-year-old studies on nitrosoproline.

The expert panelists proposed that future epidemiological studies should include diets rich and poor in nitrosamine precursors. However, measuring endogenous formation is problematic; therefore, the extensive data on exogenous formation are the pragmatic approach at this time. They must, however, be verified based on current analytics. In recent publications, CFSAN evaluated the time course of intake using a database dating to the late 1990s.

Nevertheless, collective exposure is difficult to ignore—CFSAN reported that the single highest sources of preformed nitrosamines are smoked meats, smoked fish, and grilled meats. These are also high in polyaromatic hydrocarbons and other carcinogens.

Therefore, these other sources should be considered in epidemiological studies. However, the numerical change in nitrosamine levels was less than an order of magnitude, emphasizing the reliability of use of exogenous exposure.

Q6. In the absence of data and based on identified differences in NA chemistries and reactivities, can read-across for structural similarity to related compounds be used for NAs? What are the key parameters to consider when conducting (Q)SAR assessments for NAs?

There has been much progress in in silico modeling and prediction for chemical structural similarity. The diversity of nitrosamines' behavior in vitro and in vivo has raised questions as to the reliability of (Q)SAR to predict nitrosamine mutagenicity and/or carcinogenicity. In general, the advantages of in silico approaches, and (Q)SAR modeling in particular, are that they are rapid, inexpensive, and do not use animals. However, this is an inference/prediction and so of less certainty than experimental data, and there is a need for in-depth expertise to conduct read-across.

Read-across by definition is inference of the activity or toxicity and even potency of a data-poor or no-data molecule from a similar data-rich molecule(s). For read-across there are three issues to consider for nitrosamines. First, compared to other chemicals, much data are available on mutagenicity and carcinogenicity for nitrosamines. Such data on >140 nitrosamines may be found in the U.S. EPA CompTox Chemical Dashboard and other resources, such as ChEMBL and PubChem. Whether this information is in a useful format, i.e., can be easily retrieved and used, requires further consideration.

The second issue relates to molecular similarity, a highly subjective concept. Regulatory agencies (EPA, European Food Safety Authority, European Chemicals Agency, and the Organization for Economic Cooperation and Development) have published guidances on this matter. For nitrosamines there is a need for sub-categorization to enable discrimination, which requires knowledge of their reactivity and chemistry. The knowledge can be recorded as structural and potency alerts. Such alerts can be based on chain length, alpha/beta substitution around the nitrosamine functional group, and the presence of electron-withdrawing groups.

The third issue is justification of the acceptability of predictions from approaches such as read-across. In some cases, experimental data may be needed to support the outcome. More evidence is needed to read-across a negative nontoxic or low-toxicity

compound, which is similar to a negative result by Ames test, but there is greater acceptance of the read-across prediction of a positive outcome.

(Q)SAR is a quantitative prediction based on the physicochemical properties of the molecule or its structural descriptors. For nitrosamines, (Q)SAR models should be built with nitrosamines as the largest group using mutagenicity data from the CPDB. A good understanding of reactivity is needed and ultimately the models should relate to MOA. A strong (Q)SAR model is one that has large variation of activity over several orders of magnitude and ideally with a large spread of data across these activities. This is not the case for nitrosamines because there are abundant data for high-potency nitrosamines but sparse data on those of low potency; there are, however, several ways around this. There is no correlation between the logarithm of the octanol-water partition coefficient (log P) and the TD_{50}, so other descriptors, usually for reactivity, are required.

An attendee asked whether molecular weight should be considered when extrapolating from one nitrosamine to another because the EMA's single value of 18 ng/day was based on a small molecule, i.e., NDEA. This was not recommended because many of the substituted side-chain large nitrosamines are metabolized and broken down to low-molecular-weight compounds; instead, a molar basis could be used. A large nitrosamine compound would not necessarily be less mutagenic or carcinogenic. One must be cautious in making such conclusions; e.g., dibutylnitrosamine is a more potent carcinogen in the bladder than the lower-molecular-weight NDMA, which is a potent liver carcinogen. There are many examples of high-molecular-weight nitrosamines that are potent carcinogens.

Dr. Bucher and his group at the National Institute of Environmental Health Sciences recently investigated nitrosamines using machine learning and Opera software, creating models based on physicochemical properties. Data for >700 compounds were obtained from the CPDB and restricted to 129 nitrosamines. The results were subject to five-fold cross-validation; the findings were similar to the above discussion—the (Q)SAR model needs more information to better discriminate high and low-carcinogenic potency nitrosamines. Therefore, a general prediction of carcinogenic potency is possible but its utility is unclear because of lack of diversity of nitrosamine database. Also, read-across validation is important, as is the lack of information on non-carcinogens.

It was suggested to determine why noncarcinogenic nitrosamines are inactive. Possible reasons include aging and interaction with DNA, but there are also metabolic issues, and

a trivial structural modification can markedly affect activity. For instance, if the tert-butyl group is attached (depending on its location relative to the nitrosamine moiety), then the compound is rendered inactive. Another example is isometrically substituted nitroso compounds; n-methyl nitroso pyridines have three isomers substituted at the 2'-, 3'-, and 4'-positions of the pyridine ring. The substitution at the 2'-position of pyridine was predicted to be mutagenic and carcinogenic whereas that at the other positions is not. This is a result of differential activation and inactivation of the isomers. Another example was cimetidine, the nitrosated form of which is mutagenic in vitro but not carcinogenic in rodents in vivo because it preferentially undergoes metabolic inactivation, emphasizing the importance of metabolism. It was reiterated that data on noncarcinogenic nitrosamines should be considered and why they are inactive needs to be determined. Much work on nitrosation was done by Richard Loeppky, who showed that certain structures are easily nitrosated; e.g., several tertiary amines. The WHO NAP test is a crude but easy-to-use assay of nitrosability.

Another aspect to consider in modeling is polarity. Nitrosoproline is an non-metabolized and non-carcinogenic nitroso compound that is excreted unchanged. However, whether (Q)SAR and read-across can predict biological activity is unclear.

Attendees' questions are discussed below.

Q1. The terms SAR and (Q)SAR are used interchangeably, they are approaches to assess risk based on structural analysis: if a nitrosamine can be mapped to major groups with adequate data then one that is data-poor can be mapped to that specific classification based on structure, what is the experts' opinion on such structural space for nitrosamines?

A1. SAR is similar to read-across, there is an ad hoc group led by Lhasa and LeadScope with members from academia and industry working on this topic. They are attempting to identify such structural groups and assess their effects on potency. The potential for predictability is referred to as a trend analysis. As long as there is a rational way of grouping molecules, this is achievable.

Q2. Along with (Q)SAR assessment, can we consider metabolism assessment of complex nitrosamine impurities, which would account for bioactivation of nitrosamine into reactive diazonium ion that causes carcinogenicity? For example, is a nitrosamine that converts easily to a diazonium ion more potent?

A2. Yes, it is possible to develop models for bioactivation. These could be complex if they are to be used computationally but must be able to calculate conversion to reactive diazonium ion. However, it is unclear if the data needed to build such a model are available. The answer to the second part of the question is yes.

Q3. Should SAR/read-across focus on carcinogenicity endpoint or consider separately the mutagenicity metabolic activation calculation by activated nitrosamine, or repair of alkylated DNA?

A3. Yes, generally it can be used for any other endpoint. The main issue is how will such information be used? Also, read-across and SAR require reliable data to determine similarity and dissimilarity between molecules. For complex endpoints such as carcinogenicity, this may not be possible. For less-complex endpoints—such as in vitro mutagenicity metabolic activation and alkylation—we are eliminating much variability, such as toxicokinetics (determined in vivo).

Q7. Nitrosamines can be formed during manufacturing of the active pharmaceutical ingredient (API) and/or DP. What are possible approaches to reduce nitrosamine formation during manufacturing? Can nitrosamines be eliminated completely from API and/or DP?

According to Dr. King's presentation on the contamination incident, nitrosamines as impurities can form at various stages of DS or DP manufacturing, during storage and packaging, and over time as degradants. Therefore, their detection, presence, and mitigation have been challenging both to regulators and drug manufacturers. We have learned much in the 2 years since the contamination incident was reported, but we continue to be surprised as to the ways in which nitrosamines are formed. Our understanding of all possible formation pathways continues to evolve. Drug structures and chemistries are complex, control and deep knowledge of each step in synthesis is critical, and drug manufacturers must think holistically. The process must be thought through and every possibility considered even when it's not predicted. If there is risk of nitrosamine formation, a detection strategy must be in place.

Nitrosamine contamination may not be related to the drug synthesis pathway, as was the case with the ARB, valsartan. For valsartan the issue was the solvent, dimethylformamide, which was heated to high temperatures, promoting formation of dimethylamine. The latter underwent a quenching reaction in the presence of nitrite,

which was added for a different purpose, leading to formation of NDMA. Therefore, this was a side reaction of a side reaction, not direct contamination of the DS or DP.

Another possible source is the supply chain, if drug manufacturers use recovered or recycled solvents that contain nitrosamines and then re-used in drug manufacturing. Another aspect is the stability of the drug and/or drug formulation. In some cases, nitrosamine amounts increased over time due to instability, e.g., ranitidine. An unexpected source is nitrocellulose in packaging or printing. Nitrocellulose is a strong nitrosating agent, which was identified and addressed in Europe in the cosmetics industry.

Nitrosation can occur at any step of a chemical reaction under the right conditions, i.e., in the presence of a secondary or tertiary amine, nitrite, or nitrous acid. In such cases, steps must be taken to eliminate the process or minimize nitrosamine formation by adequate purge and mitigation methods with verification by testing. When eliminating a step in which nitrosamines are formed is not possible, attempts should be made to move it earlier in synthesis and perform purging prior to production of the API.

The formation of nitrosamines can be minimized by not using a secondary or tertiary amine plus an acid, e.g., nitrous acid, in the same step of manufacturing or by eliminating one of these sources. Even when these sources are not in the same step, they could be several steps later and lead to the surprise of nitrosamine being formed. Another potential source is APIs containing residual amines put through a fluid bed drier; this is similar to malting for making beer. Nitrogen oxides are present in the environmental air (not purified air) during the malting process, which results in formation of nitrosamines; this relates to awareness of the method. Good science and manufacturing practices must always be followed. If mitigation is difficult, a multidisciplinary (pharmacology/toxicology, clinical, drug shortage, and compliance) benefit-risk assessment is needed to assess the feasibility of reducing nitrosamine contamination. Multiple aspects need to be considered, such as the patient population, the medical necessity, the balance between risk from taking a contaminated drug vs. the benefit of that drug in treating the disease.

We are working with international regulators to ensure consistent messaging and provision of safe drugs globally. Our focus is to inform about risk, and there are much data supporting that nitrosamines are potent mutagenic carcinogens in several animal species and in human. Reliable carcinogenicity data are used to inform on the safe

intake level of a drug. In the absence of such data, we use surrogate and similar molecules to determine an acceptable and safe intake limit. The goal is to eliminate nitrosamines from drugs, and with sufficient time and effort it can be achieved. In most cases, nitrosamines can be eliminated by modifying the synthetic pathway, removing a source, not using recovered solvents, and adequate purging and testing. In other cases, elimination of nitrosamines may require considerable resources and effort.

Because analytical methods continue to improve in sensitivity, very low levels of nitrosamines can be detected and so it may not be possible to completely eliminate nitrosamines from drugs. The risk-to-benefit ratio will be determined using all data available to date as well as the findings of research on the knowledge gaps identified in this workshop. Several experts emphasized that drug manufacturers must fully implement GMP and have full knowledge of their process. The qualifications of staff involved in manufacturing must also be checked.

In most cases, nitrosamines in drugs can be eliminated by GMP, good/safe chemistry, modification of the synthetic route, elimination of sources of nitrosamine formation, and a holistic understanding of the entire manufacturing process. If it is not possible to eliminate nitrosamines from drugs, a risk-benefit analysis is needed. In some cases, assessing risk may be the appropriate approach than using resources. In such cases, the patient population, drug shortages, and clinical alternatives should be considered. Advancements in manufacturing and technologies enabled the food industry to almost eliminate nitrosamines. Therefore, this can also be achieved for drugs but will take time and require collection of extensive data.

SUMMARY

The history of discovery and early research of nitrosamines as potent mutagenic carcinogens was discussed by Dr. Eisenbrand. Interest in nitrosamines faded somewhat from the mid-1990s until almost 3 years ago when they were detected as impurities in drugs. Dr. Eisenbrand presented an overview of the biological activities, metabolic activation, chemical structures of nitrosamines, as well as human exposure to nitrosamines from food, consumer products, drugs, and occupational sources. He also described their formation by nitrosation of secondary amines and from primary and tertiary amines, and their kinetics. NNN, NNA, and NNK are formed from nicotine in tobacco; the latter is responsible for a number of human cancers. The presence and potential formation of nitrosamines in cosmetics and personal care products as well as

occupational exposure by dermal and inhalation routes and their mitigation were presented. The concept of safe (non-carcinogenic) amines as a solution to eliminate nitrosamines as impurities in drugs was proposed. Mitigation of nitrosamines in food by, for example, adding ascorbic acid, tocopherol, and decreasing nitrous oxide in smoked foods, the kilning of beer and sources of nitrosamines from packaging were presented.

Endogenous formation of nitrosamines was explained beginning with the initial research carried out in the late 1960s and finishing in the 1990s. Endogenously formed nitrosamines, nitrites, and nitrates are metabolically related and undergo interconversion via reduction, oxidation, and cycling back to reactive nitrosamines in blood and tissues before being excreted as urinary nitrates. Endogenous formation of nitrosamines from various drugs was also described. To reliably determine risk, quantitative data on their endogenous formation and an understanding of the pharmacokinetics of nitrosamines are imperative. Currently, it is unknown if endogenous formation of carcinogenic nitrosamines exceeds, equals, or is less than the levels in drugs. The expert panelists emphasized the need for this information and urgently encouraged experimental work in this area.

Classifying nitrosamines would facilitate risk assessment and subgrouping based on potency and reactivity. However, the majority of nitrosamines are potent carcinogens, as shown in studies involving 40 animal species; few are non-carcinogenic. Such a vast range of potency hampers their classification. Cancer induction is cumulative although some nitrosamines can cause cancer after single exposure. Factors such as dose rate, latency to tumor induction, adducts formed, DNA-repair capacity, enzyme depletion, reactivity, and stability of reactive intermediates are important and differ among nitrosamines. The expert panelists discussed nitrosamine classification; the consensus was they should not be classified at this time because of the absence of necessary data on reactivity, DNA binding, and metabolism in human and how it differs from animals.

The above notwithstanding, a number of approaches to classification were suggested. The simplest was classification into carcinogens and non-carcinogens based on available information. However, classification based on TD_{50} values from the CPDB was considered the optimum approach at present. Although generally the BMDL would be preferred because it is based on dose response modelling using multiple doses for evaluation. The European Scientific Committee on Consumer Safety (SCCS) performed such modelling to evaluate the risk posed by nitrosamine contamination in cosmetics (SCCS 2012). Other suggestions included bucketing nitrosamines into TD_{50} value ranges

of <1, 1–10, and 10–100 mg/kg. Classification can be based on chemical reactivity, though there are no data on the correlation between reactivity and potency. Polarity and (Q)SAR/chemical structure were also discussed. Modelling would be based on TD_{50} values in conjunction with expert knowledge and in some cases supplemented with experimental data. (Q)SAR modelling is based on predictions using estimated parameters that have not been verified for nitrosamines; therefore, caution is needed. The single value of 18 ng/day implemented by EMA was acknowledged, however, this is a highly conservative approach, being based on the most potent nitrosamine, NDEA. Two interesting proposals were Bayesian probabilistic modelling of data together with SAR and an approach conceptually similar to the TTC paradigm in ICH M7 but specific to nitrosamines. The expert panelists recommended incorporating safety factors when determining risk in children, patients with enzyme deficiency, and other sensitive populations.

Applying LTL as in ICH M7 to drugs contaminated with nitrosamines indicated for short durations is being discussed by international regulators to prevent recall of drugs and allow continued use by patients when benefit outweighs risk. However, the LTL approach for nitrosamines is not accepted globally. From the FDA's perspective, this is because nitrosamines are in the CoC, which comprises chemicals of high mutagenic and carcinogenic potencies that are strictly controlled in drugs. The shape of the dose response for such chemicals is unknown when extrapolating from high to low doses; prior experimental work suggests it to be linear with no threshold even at the lowest doses. The lack of a threshold is supported by data showing the linearity of DNA adduct formation over a dose range. In place of the LTL approach, the FDA implements a flexible approach that allows values higher than the AI as interim limits yet maintaining a 1:100,000 cancer risk.

Similarly, the expert panelists had diverse opinions on LTL. Studies have shown varied dose responses for carcinogenicity among nitrosamines—some are linear, curvilinear, sigmoidal at low dose, gradual and in some cases show an abrupt transition to toxicity. The cancer rate is independent of age, e.g., the same dose of NDEA administered to animal species of different life expectancies resulted in identical rates of tumor development. Application of the LTL approach as per ICH M7, therefore, is inappropriate because the reliability of the models that extrapolate from long to short durations is unknown with respect to their sensitivity, shape of the dose response, and threshold vs. linear. However, based on the need for safe drugs, the expert panelists indicated that

the LTL approach could be considered for nitrosamines. However, using the LTL is not as simple as stating that the effect of high exposure for a short duration is similar to that of low exposure for a long duration. The expert panelists urged further studies of the factors involved.

When more than one nitrosamine is found in a drug, the expert panelists agreed that their effects would be additive. With reference to EFSA's approach for mixtures—if one of the chemicals is a carcinogen then the most conservative AI is used irrespective of the presence of mutagens or non-mutagens.

DNA adducts were indicated by the expert panelists as the best biomarkers of exposure for nitrosamines because they can be reliably quantified using highly sensitive analytical methods. They indicated that there are no biomarkers for risk assessment because the MOA must be known but this is not the case for nitrosamines. Also necessary is verifying the source of these adducts to track back to the nitrosamine from the drug, i.e., separation of adducts from food and formed endogenously should be considered. Whether risk assessment should be based on exposure from contaminated drugs alone or consider total exposure was discussed. Based on the discussion there were good arguments for each response. Ultimately, the amount in drugs may not add much to the overall risk; however, this is unknown at present. Therefore, in the absence of needed data, communication on risk should take into consideration all important factors.

The expert panelists indicated that the structures of nitrosamines must be included in risk assessment and that a blanket assessment in which all nitrosamines are placed at the same risk level is not appropriate. They emphasized that this could be mitigated by eliminating such nitrosamines and following GMP. Exposure to low levels of nitrosamines in drugs cannot be justified because nitrosamines are also found in foods; noted that content in food is at ≤1 µg/day and as low as 0.4 µg/day. The expert panelists stated that nitrosamines can be eliminated from drugs by using safe amines and good chemistry and evaluating each step of the synthetic and manufacturing processes (including un-anticipated pathways), as well as post-market monitoring such as in packaging and storage. Moreover, if there is potential for formation from a side reaction, or if a solvent or a nitrosating agent is used, then a control strategy must be in place and the nitrosamine must be identified.

Steps known to form nitrosamines should be avoided, by moving them earlier in the synthetic or manufacturing pathway when possible. In most cases nitrosamines can be

eliminated by modifying the synthesis, removing the source, and adequate purging and control. However, if elimination is complicated and resource intensive, a risk assessment can be conducted using all information available. Nevertheless, it was emphasized repeatedly by the expert panelists that nitrosamines can be eliminated from drugs by implementing GMP, smart chemistry, and awareness of the manufacturing process, although this may not be possible in a few cases. Elimination of nitrosamines from drugs should not be more difficult than their near elimination from foods.

The extensive experimental data collected over the past 20 years have been used for in silico modelling to predict outcomes. Mutagenic impurities can be qualified as described in ICH M7 when two (Q)SAR models render negative predictions. Nitrosamines are in the CoC under M7 and cannot be qualified by (Q)SAR assessment. The expert panelists deliberated on potential development and use of (Q)SAR models specific for nitrosamines and utilize expert knowledge for read-across assessment. For appropriate read-across, nitrosamines must be subcategorized based on their reactivity and chemistry, which is at present available for few nitrosamines. A negative prediction by (Q)SAR and read-across justification may require experimental testing, e.g., by new approach methodologies.

There must be a good understanding of reactivity and ultimately the models should relate back to MOA; however, data on the MOA and reactivity of nitrosamines are lacking. A reliable model requires good data and a broad range of carcinogenic activity; however, the majority of nitrosamines are potent carcinogens. Much work and collaboration among regulators as well as industry and academia is focusing on building appropriate (Q)SAR models for nitrosamines. It was suggested to use available information on noncarcinogenic nitrosamines and investigate mechanistic questions to explain their lack of activity. (Q)SAR models for nitrosamines are complicated by their diverse reactivity, stability, and behavior in vivo and in vitro; a minor structural modification can markedly alter activity, hampering the making of predictions in the absence of experimental data.

The majority of nitrosamines are mutagenic and positive by standard Ames test. However, in some cases the standard Ames test is negative but positive using different test conditions, such as pH and use of hamster S9. The Ames test is the first step in assessing the mutagenicity of a chemical for regulatory purposes. The expert panelists agreed that a negative Ames test is not in itself adequate to qualify the non-mutagenicity of a nitrosamine. A modified Ames test using different conditions should

be conducted; if the results are negative an in vivo mutagenicity assay should be performed. If the latter is also negative, expert knowledge and read-across assessment must be conducted in support of the negative mutagenicity outcome. Thorough assessment of mutagenicity is important before concluding the non-mutagenicity of a nitrosamine; some nitrosamines are non-mutagenic but carcinogenic. The expert panelists noted that the in vivo assays are mutagenicity tests for hazard identification and not applicable for cancer risk assessment. Therefore, because 2-year bioassays are infeasible, an in vivo animal model of carcinogenicity may be needed in the absence of reliable read-across via expert knowledge to a data-rich surrogate nitrosamine.

CONCLUSIONS

Nitrosamines are potent mutagenic carcinogens whose presence in drugs can in most cases be avoided by implementing good science and GMP, inspecting and evaluating every step of manufacturing, and post-market monitoring of formation and contamination. A negative Ames test is inadequate to qualify a nitrosamine as non-mutagenic. A follow-up test under modified optimal conditions is needed; if negative, an in vivo gene mutation test should follow. However, qualifying a nitrosamine as a non-mutagen does not necessarily indicate non-carcinogenicity; therefore, an alternative in vivo bioassay may be required. Caution was expressed over the LTL approach (as in ICH M7) for nitrosamines based on their diverse behaviors in vivo and in vitro, unknown dose response shape when extrapolating from long to short durations, and linear no-threshold dose response. However, to prevent drug shortages at this time other approaches for risk assessment can be applied to accept a higher AI for drugs indicated for short durations as long as a cancer risk of 1:100,000 is maintained. Multiple nitrosamines in a drug should be considered to have additive effects and the most conservative approach must be used for risk assessment. There are good arguments for use of total exposure and exposure from drugs only for risk assessment. Because of the lack of important information on endogenous formation, risk assessment based on exposure from drugs only is appropriate. (Q)SAR modelling specific to nitrosamines based on TD_{50} values is needed and must incorporate expert knowledge. TD_{50}, not BMDL, is optimal for risk assessment. NAP testing can be used to verify nitrosation to support risk assessment.

RECOMMENDATIONS OF THE EXPERT PANELISTS FOR FURTHER RESEARCH

The following research topics need to be addressed for reliable assessment of the cancer risk of nitrosamines with the ultimate goal of their elimination from drugs:

- Determination of endogenous formation.
  - Human epidemiological studies with designs incorporating diets rich and poor in nitrites, nitrates, and nitrosamines.
  - PBPK-based models to estimate endogenous exposure.
  - Prediction of nitrosation (measurement of urinary excretion of metabolites [e.g., nitrosoamino acids as surrogates]; data on distribution and excretion).
- Exogenous exposure—update current data.
- Measurement of DNA adducts as biomarkers of exposure in human; establishment of surrogate biomarkers of exposure as well as activating/deactivating biotransformations (e.g., hemoglobin adducts for long term and urinary mercapturic acids for short term); biomarkers will provide supportive information. Assessment of MGMT and other repair enzymes, such as those involved in excision repair in animals, and comparison to human; use sensitive analytical methods, verify the sources of adducts.
- Investigation of reactivity, stability, and nitrosation; correlation of reactivity to the carcinogenic potency of nitrosamines.
- Development and validation of sensitive analytical methods and monitoring for absence of artefacts.